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Different sources of reduced carbon contribute to form three classes of terpenoid emitted by *Quercus ilex* L. leaves

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ABSTRACT **_Quercus ilex_ L. leaves emit terpenes but do not have specialized structures for terpene storage. We exploited this unique feature to investigate terpene biosynthesis in intact leaves of _Q. ilex_. Light induction allowed us to distinguish three classes of terpenes: (_i_) a rapidly induced class including α-pinene; (_ii_) a more slowly induced class, including _cis_-β-ocimene; and (_iii_) the most slowly induced class, including 3-methyl-3-buten-1-ol. Using ^{13}C, we found that α-pinene and _cis_-β-ocimene were labeled quickly and almost completely while there was a delay before label appeared in linalool and 3-methyl-3-buten-1-ol. The acetyl group of 3-methyl-3-buten-1-yl acetate was labeled quickly but label was limited to 20% of the moiety. It is suggested that the ocimene class of monoterpenes is made from one or more terpenes of the α-pinene class and that both classes are made entirely from reduced carbon pools inside the chloroplasts. Linalool and 3-methyl-3-buten-1-ol are made from a different pool of reduced carbon, possibly in nonphotosynthetic plastids. The acetyl group of the 3-methyl-3-buten-1-yl acetate is derived mostly from carbon that does not participate in photosynthetic reactions. Low humidity and prolonged exposure to light favored ocimenes emission and induced linalool emission. This may indicate conversion between terpene classes.**

Biogenic hydrocarbon emissions play an important role in determining the oxidation potential of the atmosphere (1, 2). This has stimulated research into the metabolic pathway of terpene formation in plants. Isoprene biosynthesis can be studied by measuring emission rates (3); however, this is usually not possible for monoterpenes because they accumulate in specialized ducts or glands causing emissions to be independent of synthesis (4).

Quercus spp. do not have storage structures for terpenes and most emit isoprene (5). This emission is light- and CO_2-dependent, which led to the conclusion that isoprene is made by intermediates of photosynthetic carbon metabolism. This conclusion was proved correct when isoprene and phosphoglyceric acid were shown to be labeled by ^{13}C with the same rapid time course (3). Isoprene formation from dimethyl allyl pyrophosphate is catalyzed by isoprene synthase, an enzyme that is likely to be located in chloroplast stroma (6). Therefore, all of the steps of isoprene formation from photosynthetic carbon is likely to occur within chloroplasts.

Quercus ilex, an evergreen oak widespread in the Mediterranean forests, is peculiar among oaks in that it does not emit isoprene but does emit monoterpenes, primarily as α-pinene (7). Contrary to what is observed in most monoterpene emitting plants, *Q. ilex* does not have specialized structures for terpene storage. Similar to what is found in isoprene emitting plants, α-pinene emission from *Q. ilex* leaves is light- and CO_2-dependent (8, 9) and is rapidly labeled by ^{13}C (7). These findings indicate that in *Q. ilex*, α-pinene is formed in the chloroplasts. The unique features of *Q. ilex* allowed us to explore monoterpene biosynthesis by studying emission in response to light induction and humidity changes. We found three different classes of terpenes whose relative rates of emission depended on environmental factors. By labeling with ^{13}C, we studied the origin of the terpene classes to find out if different carbon sources contribute to form the skeleton of terpenes in plants.

MATERIALS AND METHODS

Plant Material. Three-year-old *Q. ilex* plants were grown in 50 liter pots in commercial soil. Plants were grown in a greenhouse where light intensity at the canopy level was about 700 μmol quanta $m^{-2} \cdot s^{-1}$ during sunny days and the air temperature was maintained between 25 and 30°C during the day and between 15 and 20°C at night. Plants were watered every other day and fertilizer was added occasionally to the irrigation water.

Light-Induction of Monoterpene Emission, Monoterpene Composition, and Changes of the Composition in Response to Humidity. A leaf was clamped in a 0.5-liter cuvette made of Plexiglas and coated with Teflon transparent film. The leaf was exposed to a 2 liter min^{-1} flow of synthetic air by mixing N_2, O_2, and CO_2 with mass flow controllers. The partial pressure of CO_2 and O_2 was set at 35 Pa and 2 kPa, respectively. A leaf temperature of 30 ± 0.2°C was set by using thermoelectric modules and was measured with a copper-constantan thermocouple appressed to the abaxial leaf side. Air humidity was set at 60% by bubbling in water the mixture of N_2 and O_2 and then removing excess humidity in a water bath. The leaf was maintained in the dark for 1 h and was then illuminated with a bulb supplying 1000 μmol photons $m^{-2} \cdot s^{-1}$; the induction of monoterpene emission was followed. We waited 80 min to allow for a stationary emission of monoterpenes and photosynthesis. Then the light was switched off again to follow the time course of decrease in monoterpene emissions. To collect monoterpene samples, a three-way Teflon valve was placed at the cuvette air outlet. When the valve was closed, the air flowed through the infrared gas analyzer for determination of photosynthesis and transpiration as described by Loreto *et al.* (9). When the valve was open, part of the air (50 to 80 ml min^{-1}) flowed through a 15 cm × 0.3 cm i.d. glass tube filled with Carbotrap C (0.034 g) (Supelco, Bellefonte, PA) and Carbotrap (0.17 g) in series. Each air sample was collected for 5 min. Traps were analyzed by GC-MS and terpenes were positively identified by combining retention features with mass spectra (7). Traps were analyzed immediately after collection.

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Abbreviation: IPP, isopentenyl pyrophosphate.

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Authentic samples were trapped and were found not to undergo rearrangement on the trap.

In a second experiment, the leaf was maintained in the light until a stable photosynthesis, transpiration, and emission of monoterpenes were obtained. Then air humidity was reduced to 10% by decreasing the temperature of the water bath while maintaining all other factors unchanged. Sixty minutes after the humidity change, the emission of monoterpenes was again measured as well as transpiration and photosynthesis.

^{13}C Incorporation in Monoterpenes. The leaf clamped in the gas-exchange cuvette was exposed to the described light, temperature, humidity, and air flow until steady photosynthesis, transpiration, and terpene emission were reached. Then the CO_2 source with a natural abundance of ^{13}C (1.1%) was rapidly substituted by a CO_2 source with 99% ^{13}C previously prepared (7). After 60 min, the natural abundance of ^{13}C was restored by switching back to the original CO_2 source. Terpenes were trapped four times during the labeling with 99% ^{13}C and four more times after restoring the 1.1% ^{13}C natural abundance. The incorporation and the disappearance of ^{13}C in the molecular ions and in different ion fragments were analyzed to see if differential labeling of carbon atoms occurred. Each experiment was performed three times on leaves of different plants. The ion fragments are reported as mass to charge ratios (m/z), which are essentially the same as molecular weight since the charge was always presumed to be 1.

RESULTS AND DISCUSSION

Light-Induction of Monoterpene Emission, Monoterpene Composition, and Changes of the Composition in Response to Humidity. *Q. ilex* leaves emitted 14 terpenes at a detectable level (Table 1). Emissions of all of the terpenes were light-dependent but the time course of light induction allowed us to distinguish two classes of terpenes (Fig. 1). The most abundant monoterpenes were rapidly induced by light. The total terpene emission, mainly reflecting the emission of α-pinene, β-pinene, sabinene, and myrcene, reached a steady state after 30 min. This is consistent with the light induction previously reported for isoprene in *Quercus rubra* (5) and α-pinene in *Q. ilex* (9). However, the emission of *cis*-β-ocimene, and several other monoterpenes reached a steady state 70 min after leaf illumination. The induction of emissions of the ocimene class of monoterpenes was mirrored by a decrease in the emission rate of α-pinene and sabinene. The emission of 3-methyl-3-buten-1-ol continued to increase for 80 min after switching the light on. Emissions of all of the terpenes ceased following darkness indicating that the emission reflects *de novo* synthesis and not release of preexisting material.

Table 1. Composition and amount of terpenes emitted by *Q. ilex* leaves

Compound	Emission, nmol $m^{-2}s^{-1}$
α-Thujene	0.08 ± 0.02
α-Pinene	2.44 ± 0.41
Camphene	0.12 ± 0.03
Sabinene	0.67 ± 0.07
α-Pinene	1.60 ± 0.20
Myrcene	0.50 ± 0.09
β-Phellandrene	0.04 ± 0.01
α-Terpinene	0.11 ± 0.03
Limonene	0.29 ± 0.08
cis-β-Ocimene	0.27 ± 0.09
trans-β-Ocimene	0.06 ± 0.02
γ-Terpinene	0.10 ± 0.03
Para-cymene	0.28 ± 0.10
Linalool	0.22 ± 0.10
3-Methyl-3-buten-1-ol	0.38 ± 0.04
3-Methyl-3-buten-1-yl acetate	0.10 ± 0.02
Total terpenes	6.72 ± 0.99

All compounds detected were labeled when $^{13}CO_2$ was fed to the leaf as shown in Fig. 2. Average of five measurements ± standard error is presented. Measurements were carried out under ambient air (35 Pa CO_2, 2 kPa O_2), at a photon flux density of 1000 μmol $m^{-2}s^{-1}$ and at a leaf temperature of 30°C. Average photosynthesis was 6.5 ± 1.3 μmol $m^{-2}s^{-1}$.

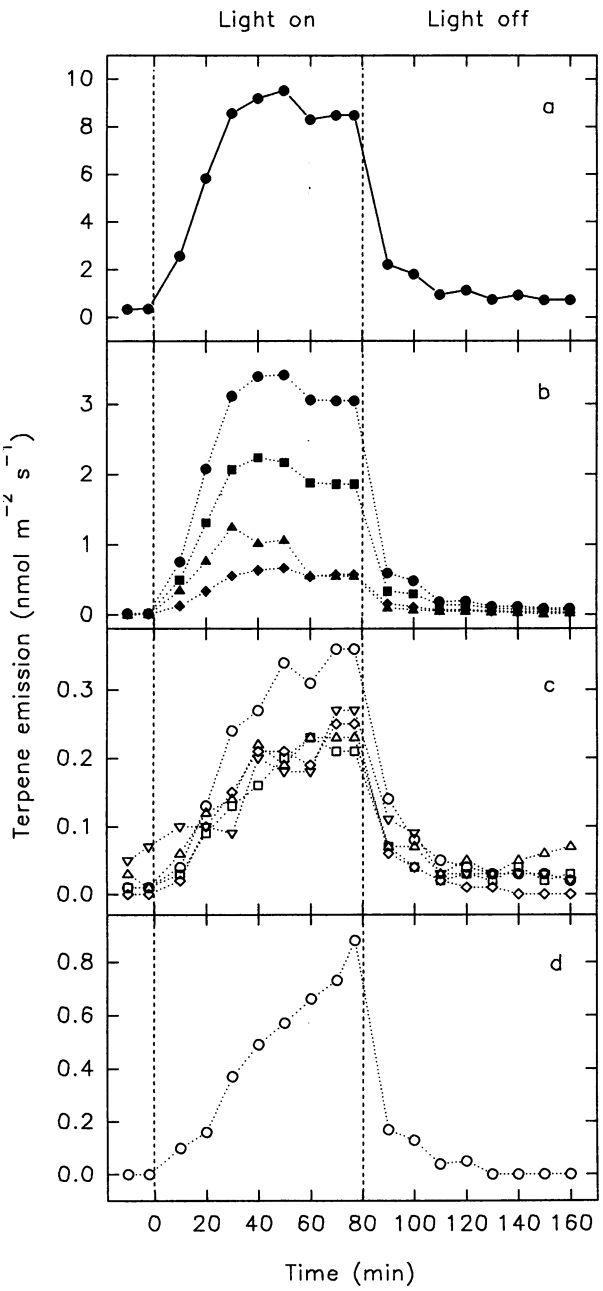

FIG. 1. Light induction of terpene emissions in *Q. ilex* leaves. The leaf was maintained in the dark for 1 h, then (at time = 0 as shown by the dashed vertical line on the left) it was illuminated with a bulb supplying 1000 μmol photons $m^{-2}s^{-1}$. After 80 min, the light was switched off (the right dashed vertical line). (*a*) The total emission of terpenes during the experiment. (*b*) The main monoterpenes emitted are shown. ●, α-Pinene; ■, β-pinene; ▲, sabinene; ♦, myrcene. (*c*) A second class of monoterpenes characterized by lower emission and slower light induction with respect to monoterpenes of *b*. ○, *cis*-β-Ocimene; □, γ-terpinolene; △, α-terpinene; ▽, para-cymene; ◇, β-phellandrene. (*d*) The light induction of 3-methyl-3-buten-1-ol is reported.

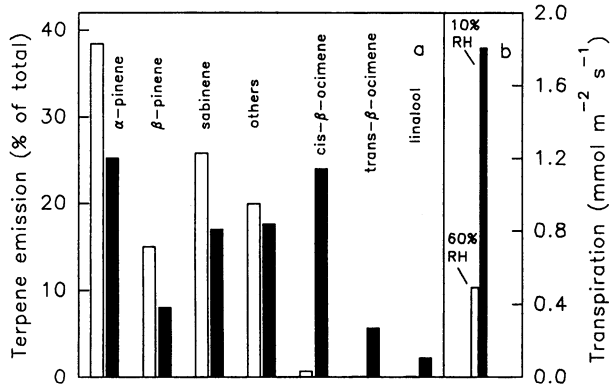

FIG. 2. Changes in the composition of the emission of selected terpenes (*a*) and transpiration (*b*) of a *Q. ilex* leaf exposed for 1 h to 60% (open bars) or 10% (solid bars) RH.

Terpene synthases can be nonspecific. As an example, limonene synthase can form α-pinene, β-pinene, or limonene (10). It is possible that all of the monoterpenes in the pinene class are made by one monoterpene synthase and that this enzyme is light inducible. The decline in the emission rate of the α-pinene class when the ocimene class was increasing after light induction could result from conversion of α-pinene class compounds (mainly α-pinene and sabinene) to ocimene class compounds. However, conversion from cyclic to acyclic monoterpene requires energy and is therefore unlikely to occur.

A reduction of air humidity from 60% to 10% did not change the total emission of monoterpenes (data not shown) but changed the composition of the emission (Fig. 2). At low humidity, terpene emission from the α-pinene class was lower while the emission of *cis*-β-ocimene was stimulated and a small emission of linalool was induced. As in the case of light induction, this finding may indicate a conversion among terpenes under different environmental conditions but could also result from physical properties of the terpenes.

Terpenes have different solubilities in water. We measured the relative water/air partition coefficients and found that α-pinene and β-pinene were less water soluble than ocimene and linalool (Table 2). This may explain the larger emission of α-pinene and β-pinene compared with the other terpenes (Table 1). Because of the hydroxyl group, which forms strong hydrogen bonds with water molecules, linalool was expected to be very low in the gas phase and almost undetectable in the emission (Table 1 and Fig. 2). We think that the increased transpiration rate at low humidity may increase emission of more water-soluble terpenes, perhaps by dragging liquid to sites where the change of phase is favored. As a general conclusion, these experiments revealed that the composition of the terpene emission is not constant but may substantially change in response to environmental changes. These findings may be useful to improve models of biogenic emissions and in assessing their oxidation potential during different seasons.

Table 2. Partition coefficients between water and air of selected monoterpenes relative to α-pinene at 20°C and one atmosphere pressure

Compound	Ratio
α-Pinene	1.00
β-Pinene	1.22
Limonene	2.04
cis-β-Ocimene	1.88
trans-β-Ocimene	1.94
Linalool	>100,000

^{13}C Incorporation in Monoterpenes. When leaves were exposed to air containing 99% $^{13}CO_2$, the labeling time course was rapid and complete for the monoterpenes of the α-pinene and ocimene classes (Fig. 3*a*). After 20 min, all of the carbon emitted as *cis*-β-ocimene was ^{13}C. Twenty minutes after returning to the ^{13}C natural abundance (1.1%), *cis*-β-ocimene emitted was unlabeled. The labeling and unlabeling time courses of *cis*-β-ocimene were similar to those reported for α-pinene (7), isoprene (3), and the photosynthetic intermediate phosphoglyceric acid (11). This supports our idea that the appearance of monoterpenes such as *cis*-β-ocimene does not represent a different biosynthetic pathway but represents either the conversion among monoterpenes or the release of terpenes with different partitioning coefficients between gas and liquid phases.

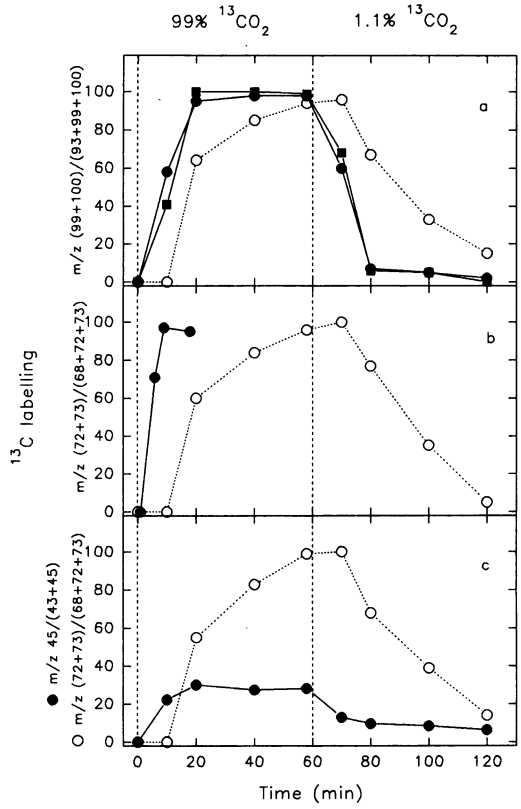

FIG. 3. ^{13}C labeling and unlabeling time course of different terpenes emitted by *Q. ilex* leaves. A leaf was clamped in the gas-exchange cuvette described in Fig. 1 and exposed to the same light, temperature, and humidity. The leaf was maintained under a 2 liter min^{-1} flow of synthetic air until steady photosynthesis, transpiration, and terpene emission were reached. At time = 0 (the dashed vertical line on the left), the CO_2 source with a natural abundance of ^{13}C (1.1%) was rapidly substituted by a CO_2 source with 99% ^{13}C previously prepared (7). At time = 60 min (the dashed vertical line on the right), the natural abundance of ^{13}C was restored by switching back to the original CO_2 source. Terpenes were trapped four times during the labeling with 99% ^{13}C and four more times after restoring the 1.1% ^{13}C natural abundance. The incorporation and the disappearance of ^{13}C in the molecular ions and in different ion fragments expressed as mass to charge ratios (m/z) was analyzed to discard the possibility that differential labeling of carbon atoms occurred. Labeling and disappearance of ^{13}C in the most abundant fragments is shown. (*a*) For monoterpenes, this is given by m/z (99 + 100)/(93 + 99 + 100). The labeling time course recalculated for α-pinene (7) (●) was compared with that measured on *cis*-β-ocimene (■) and on linalool (○). (*b*) The labeling time course was recalculated as m/z (72 + 73)/(68 + 72 + 73) for isoprene [data from Delwiche and Sharkey (3); ●] and this was compared with the labeling time course of the 5-carbon moiety of 3-methyl-3-buten-1-ol (○). (*c*) The labeling time course of the 2-carbon [m/z 45/(42 + 45) 151] and 5-carbon moieties [m/z (72 + 73)/(68 + 72 + 73) 153] of 3-methyl-3-buten-1-yl acetate is shown.

These results indicate that, as is the case for isoprene (3, 5), monoterpenes are also formed by photosynthetic carbon. It is suggested that photosynthesis supplies the carbon that becomes the five carbon compound isopentenyl pyrophosphate (IPP). All IPP needed for terpenoid synthesis is made in plastids of peppermint glandular trichomes (12). The rapid labeling is most consistent with the interpretation that both the α-pinene and the *cis*-β-ocimene groups are made from carbon that never left the chloroplast.

Linalool and the 5-carbon moieties of 3-methyl-3-buten-1-ol and 3-methyl-3-buten-1-yl acetate were labeled and unlabeled with a slower time course than α-pinene and after a delay (Fig. 3). Therefore, these compounds are made from a different source of reduced carbon than the other terpenes. The emission of linalool and 3-methyl-3-buten-1-yl acetate during light induction was small and not clearly detectable. Because of the labeling pattern, we expect that these compounds have a slow light induction, as does 3-methyl-3-buten-1-ol. Linalool synthase is only loosely related to other monoterpene synthases (13). Linalool is frequently found in flower chromoplasts where the fragrance attracts pollinators (14), but we are unaware of any reports of its synthesis in leaf chloroplasts. One explanation of these results is that there exist nonphotosynthetic plastids in *Q. ilex* leaves that rely on carbon exported from chloroplasts and that express genes normally found in chromoplasts such as linalool synthase. Aach *et al.* (15) reported that the isoprenoid *ent*-kaurene is synthesized only in nonchloroplastic plastids in wheat. The activity of geranyl pyrophosphate synthase, a key enzyme for monoterpene formation from IPP units, has also been shown in *Vitis vinifera* plastids (16). Our findings support the suggestion that an IPP translocating system exists that may play a central role in the regulation of monoterpene biosynthesis and emission (16). Similarly, the 5-carbon moiety of 3-methyl-3-buten-1-ol may come from the same carbon source as linalool.

In 3-methyl-3-buten-1-yl acetate, labeling of the acetate moiety was fast and incomplete while the 5-carbon moiety was labeled as in 3-methyl-3-buten-1-ol (Fig. 3*c*), which suggests a common origin of the 5-carbon compounds. The 2-carbon compound mostly comes from an unlabeled pool of carbon and only partially from photosynthetic carbon. The lack of labeling of the acetyl moiety indicates that there is a pool of acetate or acetyl CoA that is not closely linked to photosynthesis. Monoterpenes are not made from carbon of this pool but acetates emitted by plants may partially be formed by this carbon.

The data presented here indicate that the monoterpenes other than linalool are made from recently fixed carbon as is isoprene (3, 5). Many hypotheses have been made about why plants emit isoprene (17–19). Monoterpenes, on the other hand, are believed to play ecological roles such as chemical defense or pollinator attraction (20). Because of the similarity in environmental effects on monoterpene emission by *Q. ilex* (8, 9) and isoprene emission in *Q. rubra* (5), we suggest that these compounds may play the same physiological role; monoterpenes may protect membranes at high temperatures as suggested for isoprene (18). An alternative hypothesis is that isoprene and monoterpenes are both by-products in the metabolic pathway leading to storage of monoterpenes in specialized structures such as resin ducts or oil glands (21). Since *Quercus* spp. did not evolve these structures, terpenes are immediately emitted by *Q. ilex* leaves.

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1. Chameides, W. L., Lindsay, R. W., Richardson, J. & Kiang, C. S. (1988) *Science* **241,** 1473–1475.
2. Fehsenfeld, F., Calvert, J., Fall, R., Goldan, P., Guenther, A. B., Hewitt, C. N., Lamb, B., Liu, S., Trainer, M., Westberg, H. & Zimmerman, P. (1992) *Global Biogeochem. Cycles* **6,** 389–430.
3. Delwiche, C. F. & Sharkey, T. D. (1993) *Plant Cell Environ.* **16,** 587–591.
4. Tingey, D. T., Turner, D. P. & Weber, J. A. (1991) in *Trace Gas Emissions by Plants*, eds. Sharkey, T. D., Holland, E. A. & Mooney, H. A. (Academic, San Diego), pp. 93–119.
5. Loreto, F. & Sharkey, T. D. (1990) *Planta* **182,** 523–531.
6. Silver, G. M. & Fall, R. (1991) *Plant Physiol.* **97,** 1588–1591.
7. Loreto, F., Ciccioli, P., Cecinato, A., Brancaleoni, E., Frattoni, M., Fabozzi, C. & Tricoli, D. (1996) *Plant Physiol.* **110,** 1317–1322.
8. Staudt, M. & Seufert, G. (1995) *Naturwissenschaften* **82,** 89–92.
9. Loreto, F., Ciccioli, P., Cecinato, A., Brancaleoni, E., Frattoni, M. & Tricoli, D. (1996) *Plant Physiol.* **110,** 267–275.
10. Croteau, R. (1987) *Chem. Rev.* **87,** 929–954.
11. Canvin, D. T. (1979) in *Encyclopedia of Plant Physiology*, eds. Gibbs, M. & Latsko, E. (Springer, Berlin), pp. 368–396.
12. McCaskill, D. & Croteau, R. (1995) *Planta* **197,** 49–56.
13. McGarvey, D. J. & Croteau, R. (1995) *Plant Cell* **7,** 1015–1026.
14. Pichersky, E., Raguso, R. A., Lewinsohn, E. & Croteau, R. (1994) *Plant Physiol.* **106,** 1533–1540.
15. Aach, H., Böse, G. & Graebe, J. E. (1995) *Planta* **197,** 333–342.
16. Soler, E., Feron, G., Clastre, M., Dargent, R., Gleizes, M. & Ambid, C. (1992) *Planta* **187,** 171–175.
17. Fall, R. (1991) in *Trace Gas Emissions by Plants*, eds. Sharkey, T. D., Holland, E. A. & Mooney, H. A. (Academic, San Diego), pp. 209–216.
18. Sharkey, T. D. & Singsaas, E. L. (1995) *Nature (London)* **374,** 769.
19. Terry, G. M., Stokes, N. J., Hewitt, C. N. & Mansfield, T. A. (1995) *J. Exp. Bot.* **46,** 1629–1631.
20. Monson, R. K., Guenther, A. B. & Fall, R. (1991) in *Trace Gas Emissions by Plants*, eds. Sharkey, T. D., Holland, E. A. & Mooney, H. A. (Academic, San Diego), pp. 185–207.
21. Kleinig, H. (1989) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **40,** 39–59.